Regulation of Central Nervous System Inflammation by Anti-inflammatory Cytokines in Activated Microglia and Impairments in Aging

A Senior Honors Thesis

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Abstract

Prolonged central nervous system (CNS) inflammation is a problem because it can cause behavioral and neurological complications including depression and cognitive dysfunction. After a peripheral immune stimulus microglia, the innate immune cells of the CNS, become activated and release pro-inflammatory cytokines. Regulation of microglia by anti-inflammatory cytokines including interleukin (IL)-4, IL-10, and transforming growth factor beta (TGFβ) may be necessary to transition to a less inflammatory repair phenotype. In normal aging, however, this process may be impaired as microglia from aged animals have a prolonged and exaggerated inflammatory response and are resistant to anti-inflammatory feedback. This impairment of microglia regulation may explain the comorbidity of depression and cognitive problems with peripheral illnesses and injury in elderly people. To identify how these regulatory processes can become impaired with age, we first wanted to establish the extent to which activated microglia from adult mice are sensitive to IL-4, IL-10, and TGFβ. We report that activation of microglia *in vivo* with lipopolysaccharide (LPS), a component of gram negative bacteria cell wall, increased the expression of IL-4 receptor (IL-4Rα), but not IL-10 receptor (IL-10R1) on the surface of microglia. IL-4 and TGFβ decreased inflammatory cytokine expression and increased anti-inflammatory cytokine expression *in vitro* but IL-10 had no such effect. In adult *ex vivo* cultures, TGFβ decreased production of IL-1β and increased production of IL-4Rα; IL-4 treatment decreased inducible nitric oxide synthase (iNOS) and increased arginase expression. In aged *ex vivo* cultures, TGFβ had no significant effect on IL-1β, CX_3CR1, and IL-4Rα expression; IL-4 treatment increased IL-1β, had no effect on iNOS, and increased arginase expression. Preliminary data from intracranioventricular (ICV) injection of a TGFβ inhibitor following intraperitoneal (i.p.) LPS injection show a significant lack of social exploratory behavior at 24 h

and a tendency towards higher expression of IL-1β and IL-6 from brain slice mRNA. Expression of IL-4Rα and IL-10R1 on microglia from LPS-injected adult BALB/c mice was determined by flow cytometry. BV2 cells, a mouse microglia cell line, were treated with IL-4, IL-10, or TGFβ in the presence or absence of LPS and mRNA levels were determined using qPCR. Primary microglia cultures established from neonatal mice were treated with TGFβ and LPS and mRNA was analyzed using qPCR. *Ex vivo* cultures were established from adult or aged BALB/c mice microglia that were LPS-activated *in vivo*; mRNA was analyzed using qPCR. These findings indicate that IL-4 and TGFβ are more potent anti-inflammatory mediators for microglia than IL-10. IL-4 and TGFβ treatment redirects activated microglia towards a less inflammatory phenotype. In aged microglia, however, there is an incomplete resolution of inflammation with IL-4 or TGFβ treatment.

1. Introduction

1.1. Microglia

Microglia are the innate immune cells in the CNS and account for approximately 12% of cells in the brain. Primitive macrophage precursors from the embryonic yolk sac infiltrate the brain and differentiate into microglia (Schwarz et al., 2012). Microglia serve as the resident macrophages of the brain but they differ from peripheral macrophages in that microglia have a slower turnover rate, express little to no MHC in a resting state, and are more tightly regulated spatially and temporally. This regulation of microglia is necessary for a coordinated immune response (Aloisi, 2001).Thus, microglia act as the first line of immune defense in the brain and spinal cord. Without an immune stimulus, resting microglia survey the microenvironment. Conversely, microglia function to phagocytose, present antigens, and propagate inflammatory signals upon activation from immune and/or inflammatory stimuli. During activation, microglia exhibit an M1 or inflammatory phenotype and transition to an M2 or reparative phenotype. Regulation of microglia by anti-inflammatory cytokines may be necessary to transition to a repair phenotype. In normal non-diseased states, inflammation associated with microglia activation is transient and dissipates with the resolution of the immune stimulus. Thus, microglia propagate cytokine signals to regulate the microenvironment in the CNS.

1.2. Cytokines in the CNS

Cytokines are small polypeptide cell-signaling molecules that exhibit an immunomodulatory function. They have an important presence in the CNS because they are both secreted by and have effects on microglia. Key cytokines in the CNS that complete a role in microglia regulation

include anti-inflammatory mediators interleukin (IL)-4, IL-10, transforming growth factor beta (TGFβ) and pro-inflammatory cytokines IL-1β, IL-6, tumor necrosis factor alpha (TNFα) as well as the chemokine receptor CX_3CR1. When microglia are activated, they produce pro-inflammatory IL-1β, IL-6, and TNFα. These cytokines stimulate and sustain adaptive sickness behaviors including reduced food intake, lethargy, and social withdrawal. Sickness behaviors conserve an organism's energy for production of heat in fever and ultimately aid in the resolution of the infection (Dantzer, 2001). In normal non-diseased states, this transient up-regulation of inflammatory cytokines is beneficial to the organism. When modulation of inflammatory cytokines is misregulated, however, certain CNS complications can occur including cognitive impairments, amplified and prolonged sickness behavior, depressive-like behavior, and mechanisms that may contribute to neurodegeneration (Perry et al., 2003).

Furthermore, regulation of inflammation by anti-inflammatory cytokines may be necessary in transitioning microglia to a repair phenotype. IL-4, IL-10, and TGFβ are potent anti-inflammatory cytokines that have several functions including inhibiting cytokine synthesis, reducing macrophage activation, and promoting neuroprotection (Male et al., 2006; Spitzbarth et al., 2012). These anti-inflammatory cytokines may have the potential to redirect activated or primed microglia to a reparative M2 phenotype. Therefore, the purpose of this study was to determine the degree to which activated microglia from adult and aged mice were sensitive to the anti-inflammatory effects of IL-4, IL-10, and TGFβ.

1.3. Neuroinflammation and aging

Although inflammation modulated by adult microglia is transient, normal aging may

contribute to an exaggerated and prolonged inflammatory profile. Microglia in the aged brain may be “primed” or “reactive” to immune stimuli, express a number of M1 inflammatory markers, and exhibit de-ramified morphology (Choi et al., 2007). Following a peripheral immune stimulus, these primed microglia produce an exaggerated and prolonged inflammatory response which can produce CNS complications including cognitive impairments, exaggerated and prolonged sickness behavior, and depressive-like behavior (Barrientos et al., 2006; Abraham et al., 2008; Godbout et al., 2008; Godbout et al., 2005b). This amplified and extended inflammatory response propagated by primed microglia may explain the comorbidity of depression and cognitive problems with peripheral illnesses and injury in the elderly compared to their adult counterparts. As such, the regulation of aged microglia by anti-inflammatory cytokines is of great interest and is the focus of this thesis.

2. Methods and Materials

2.1. Animals

Adult (3-4 months old) male BALB/c mice were obtained from a breeding colony kept in barrier-reared conditions in a specific-pathogen-free facility at the Ohio State University. Mice were individually housed in polypropylene cages and maintained at 25 °C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. For age comparison, male BALB/c mice (18–22 mo) were purchased from the National Institute on Aging Specific-Pathogen-Free Colony (maintained at Charles River Laboratories, Inc., MA). The median lifespan for BALB/c mice is approximately 26 months (Miller et al., 2005). Aged mice were acclimated to the facilities for one week prior to experimentation. To investigate changes that occur from adulthood to what is considered aged, 3-4 month-old (adult) and 18-22 month-old (aged) male BALB/c mice were used. Upon arrival, mice were individually housed as described above. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

2.2. BV2 cell culture and treatment

BV2 cells were cultured in DMEM (Dulbecco's modified eagle medium) growth medium supplemented with 10% fetal bovine serum (FBS), 3.7 g/L sodium bicarbonate, 200 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, as previously described (Wynne et al., 2010). Cultures were maintained and incubated at 37 °C with 95% humidity and 5% CO_2 and growth medium was replenished every third day until confluence. Before the experiments, cells were plated at 1 x 10^5 cells/well in 24-well plates and allowed to adhere for 20 h. Immediately

before treatment, cells were washed twice with serum-free DMEM and supplemented with warm serum-free DMEM containing experimental treatments. Following treatment and incubation, cells were homogenized and RNA was isolated. For this study, cells were treated with saline or LPS (100 ng/ml), then 1 h later treated with either vehicle (0.1% bovine serum albumin [BSA]), IL-4 (20 ng/ml), IL-10 (10 ng/ml), or TGFβ (1 ng/ml) and incubated for 3 h.

2.3. Primary microglia treatment and culture

Microglia cultures were established from neonatal mice as previously described (Godbout et al., 2004). In brief, whole brains were aseptically removed and mechanically dissociated after a 15 min trypsinization (0.25% trypsin) and passed through a 70 μm nylon mesh, washed twice in PBS, and plated on poly-L-lysine coated 162 cm^2 culture flasks in DMEM growth medium supplemented with 20% FBS, 3.7 g/l sodium bicarbonate, 200 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin. Mixed glia cultures were maintained at 37 °C with 95% humidity and 5% CO_2 and growth medium was replenished every third day until confluence. Mixed glia cultures were shaken at 120 rev/min and 37 °C for 3.5 h to harvest microglia from the confluent cell layer. Cells were collected, counted by trypan blue staining on a hemocytometer, and plated at a density of 1 x 10^5 cells/well per 500 μl on poly-L-lysine coated 24-well plates. After 48 h, microglia were washed twice with serum-free DMEM medium (growth medium without FBS) and supplemented with warm serum-free DMEM containing experimental treatments. For this thesis, cells were treated with saline or LPS (10 ng/ml) for 1 h, then treated with either vehicle (0.1% BSA) or TGFβ (1 ng/ml).

2.4. Ex vivo treatment and culture

Enriched microglia were washed with PBS and counted by hemocytometer. Cells from each animal were then plated at 1 x 10^5 cells/well into two wells of a poly-L-lysine coated 24-well plate in serum-free DMEM medium. Cells were incubated at 37 °C with 95% humidity and 5% CO_2 and allowed to adhere for 1 h before experimental treatments. After treatment and incubation, cells were homogenized and RNA was isolated. For this report, adult (3-6 mo) and aged (18-22 mo) BALB/c mice were euthanized 4 h after i.p. LPS (0.33 mg/kg). The isolated enriched microglia were treated with either vehicle (0.1% BSA), IL-4 (20 ng/ml), or TGFβ (1 ng/ml) for 3 h.

2.5. Microglia isolation

Microglia were isolated from whole brain homogenates as described previously (Henry et al., 2009). In brief, brains were homogenized in phosphate buffer solution (PBS, pH 7.4) and passed through a 70 μm nylon cell strainer. Resulting homogenates were centrifuged at 600 g for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll (GE-healthcare, Uppsala, Sweden) at room temperature. A discontinuous Percoll density gradient was layered as follows: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 minutes at 2000 g and microglia were collected from the interface between the 70% and 50% Percoll layers (Frank et al., 2006b). Cells were washed and then re-suspended in sterile PBS. The number of viable cells was determined using a hemocytometer and 0.1% Trypan blue staining. Each brain extraction yielded approximately 3 x 10^5 viable cells. The Godbout lab has previously characterized these cells as approximately 85% CD11b+/$CD45^{low}$ microglia with less than 1% CD11b+/$CD45^{high}$ macrophages (Henry et al., 2009). These relative percentages of cells do not

change with age or LPS (Henry et al., 2009). Based on this previous characterization, cells isolated by Percoll density separation are referred to as "enriched microglia".

2.6. RNA isolation and qPCR

RNA was isolated from BV2 cells, primary microglia, a coronal brain slice, or enriched microglia. For the BV2 cells, coronal brain slice, and primary microglia, total RNA was isolated using the Tri-Reagent protocol (Sigma, MO). For enriched microglia, RNA was isolated using the PrepEase kit (USB, OH). In all RNA isolation procedures, RNA concentration was determined by spectrophotometry and RNA was reverse transcribed to cDNA. Quantitative polymerase chain reaction (qPCR) was performed using the Applied Biosystems (Foster, CA) Taqman gene expression assay as previously described (Godbout et al., 2005b). In brief, cDNA was amplified by real time PCR where a target cDNA and a reference cDNA (glyceralde-hyde-3-phosphate dehydrogenase [GAPDH]) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). Fluorescence was determined on an ABI PRISM 7300 sequence detection system (Applied Biosystems, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference from saline controls. In the microglia *ex vivo* cultures established from the brains of adult or aged mice, fold change results are expressed as fold change from the age-matched saline controls.

2.7. Cannulation surgeries and intracranioventricular injections

The intracranioventricular (ICV) cannulation was performed as previously described with a

few modifications (Godbout et al., 2005a). In brief, mice were deeply anesthetized using ketamine and xylazine (100 mg and 10 mg/kg BW i.p., respectively) and the surgical site was shaved and sterilized. Mice were positioned in a stereotaxic instrument so that the plane formed by the frontal and parietal bones was parallel to the table top. An incision, 1.5 cm in length, was made on the cranium to reveal bregma and a 26-guage stainless-steel guide cannula was placed in the lateral cerebral ventricle using the following stereotaxic coordinates: lateral 1.2 mm, anterior-posterior 0.5 mm to bregma, and 2mm from the dura mater. Two anchoring cranial screws were inserted adjacent to the cannula and the cannula was secured with cranioplastic cement. A dummy cannula was inserted in the guide cannula to prevent occlusion and infection. Mice were injected subcutaneously with Buprinex (111 g/kg BW) following surgery. Mice were provided a minimum of 7 d to recover before any treatment was administered. For ICV injection into the lateral ventricle, the cannula was connected with sterile tubing to a Hamilton syringe and injections were administered using a KS Scientific precision syringe pump. Vehicle (50/50 PBS/DMSO) or TGFβ inhibitor (SB 431542, Sigma Aldrich, St. Louis, MI) was injected ICV into the lateral ventricle at a dose of 2.5 nmoles in 2 μl at a rate of 1 μl/min.

2.8. Flow cytometry

Enriched microglia were assayed for surface antigens by flow cytometry as previously described (Henry et al., 2008, 2009). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody. Next, enriched microglia were split for staining using two separate panels of antibodies. Cells for Panel 1 were incubated with rat anti-mouse antibodies (eBioscience, CA): CD11b-APC, CD45-PerCP-Cy5.5, and IL-10R1-PE. Cells for Panel 2 were incubated with rat

anti-mouse antibodies (eBioscience, CA): CD11b-APC, CD45-PerCP-Cy5.5, and IL-4Ra-PE. Expression of these surface receptors was determined using a Becton-Dickinson FACSCaliber four color cytometer. Twenty thousand events were recorded and microglia were identified by CD11b+/CD45^{low} expression (Wohleb et al.,2011). For each antibody, gating was determined based on appropriate negative isotype stained controls. In age comparisons of IL-4Rα, separate isotypes were used for adult and aged mice to control for the increased non-specific staining detected in microglia from aged mice. Flow data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

2.9. Social exploratory behavior

To assess the motivation to engage in social exploratory behavior, a novel juvenile was introduced into the test mouse's home cage for a 10 min period at 0 h, 4 h, 8 h, and 24 h post LPS. Behavior was videotaped and the time the test mouse engaged in social investigation was determined from the video records by a trained observer who was blind to the experimental treatments. Baseline social behavior was measured at 0 h for all experimental treatments. Social behavior was determined as the amount of time that the experimental subject spent investigating the juvenile. Results are expressed as percent of baseline (0 h) social behavior.

3. Results

3.1. IL-4 treatment down-regulated M1-related genes and up-regulated M2-related genes in BV2 microglia

Activated BV2 microglia cultures were treated with IL-4 to understand its effects on the inflammatory profile of microglia *in vitro.* In activated BV2 microglia, IL-4 treatment significantly decreased the expression of inflammatory markers iNOS and IL-1β ($p < 0.0001$ for each) and increased anti-inflammatory markers arginase and suppressor of cytokine signaling 1 (SOCS1) ($p < 0.02$). In this experiment, arginase and SOCS1 were strongly induced at 30-fold up-regulation in arginase and 90-fold increase in SOCS1 production (figure 1). These results show that IL-4 is able to resolve inflammation and transition activated microglia to an M2 phenotype.

3.2. TGFβ decreases inflammatory cytokine expression in activated BV2 and primary microglia

To understand the effects of TGFβ treatment on LPS-activated BV2 and primary microglia, the mRNA expression of a panel of anti- and pro-inflammatory cytokines and receptors was analyzed. CX_3CR1 and IL-4Rα were chosen as anti-inflammatory markers; IL-1β, IL-6, and TNFα were selected as markers of inflammation. TGFβ treatment on LPS-activated BV2 microglia significantly up-regulated CX_3CR1 ($p < 0.0001$), trended to increase IL-4Rα ($p < 0.1$), and decreased IL-1β, IL-6, and TNFα ($p < 0.001$ for each) (figure 2a). In the activated primary microglia mRNA profile, TGFβ treatment significantly increased CX_3CR1 ($p < 0.0001$) and IL-

4Rα ($p < 0.001$), and decreased expression of IL-1β ($p < 0.0001$) and IL-6 ($p < 0.005$) (figure 2b).

3.3. IL-10 failed to fully promote an M2 profile in activated BV2 microglia

Activated BV2 microglia were treated with IL-10 to understand its effect on several markers of inflammation. IL-10 treatment of activated BV2 microglia increased iNOS expression ($p < 0.04$), had no effect on IL-1β and arginase, and increased SOCS3 mRNA ($p < 0.02$) (figure 3). Although the anti-inflammatory marker SOCS3 was increased 8-fold, it was not increased to the extent to which IL-4 treatment increased SOCS1 (90-fold). These results show that IL-4 and TGFβ may be more potent anti-inflammatory mediators than IL-10 on activated microglia.

3.4. Peripheral LPS injection increased surface expression of IL-4Rα, but not IL-10R1, on adult microglia

After determining that IL-4 had more potent effects on the inflammatory profile of activated microglia than IL-10, the surface expression of IL-4 receptor (IL-4Rα) and IL-10 receptor (IL-10R1) on LPS-activated mouse microglia was analyzed by flow cytometry. Both 4 h and 24 h after LPS treatment, enriched microglia significantly increased IL-4Rα surface expression (main effect of LPS, $p < 0.0001$ at 4 h, $p < 0.003$ at 24 h). IL-10R1 expression, however, was unchanged compared to saline controls 4h and 24 h post LPS (figure 4). This difference in expression of receptors may explain why IL-4 treatment showed a more potent anti-inflammatory effect on activated microglia compared to IL-10 treatment.

3.5. Ex vivo IL-4 treatment lowered inflammation in adult, but not aged, microglia

To investigate the extent to which inflammation is regulated in adult and aged microglia, enriched microglia were treated *ex vivo* with IL-4 and mRNA was analyzed using qPCR. IL-4 treatment *ex vivo* on adult enriched microglia had no effect on IL-1β, increased arginase ($p < 0.01$), and decreased expression of iNOS ($p < 0.0002$) (figure 5). Aged enriched microglia, however, exhibited an incomplete resolution of inflammation with IL-4 treatment. In aged mice microglia, IL-4 treatment had no effect on iNOS, increased IL-1β expression ($p < 0.05$), and increased arginase ($p < 0.003$) (figure 5). Although *ex vivo* IL-4 treatment on aged microglia has some anti-inflammatory effect with increased arginase, it does not produce the same reduction in inflammatory markers seen in adult *ex vivo* cultures.

3.6. Peripheral LPS injection increased surface expression of IL-4Rα on adult, but not aged, microglia

After establishing the anti-inflammatory effects of IL-4 in adult and aged activated microglia, it was necessary to investigate the expression of IL-4Rα. Therefore, the expression of IL-4Rα on the surface of adult and aged mouse microglia was analyzed using flow cytometry. Enriched microglia from adult mice showed a significant increase in IL-4Rα expression ($p < 0.0001$) in adult mice 4 h and 24 h after peripheral LPS injection. This increase, however, was absent in enriched microglia of aged mice both 4 h and 24 h post LPS (figure 6). This absence in IL-4Rα expression on the surface of aged microglia may explain how IL-4 treatment presents an incomplete resolution of inflammation in aged microglia compared to adults.

3.7. Resolution of inflammation is incomplete in aged microglia with TGFβ treatment

After the effects of TGFβ treatment were determined *in vitro*, the effects of TGFβ treatment *ex vivo* were examined by analyzing mRNA expression of anti- and pro-inflammatory markers in adult and aged LPS-activated enriched microglia. In adult microglia, TGFβ treatment alone significantly increased CX_3CR1 ($p < 0.01$) and IL-4Rα (0.001), and decreased IL-1β mRNA expression ($p < 0.01$). In addition, adult LPS-activated microglia trended to up-regulate IL-4Rα ($p < 0.2$) and trended to down-regulated IL-1β ($p < 0.1$) (figure 7). *Ex vivo* TGFβ treatment on aged enriched microglia, however, had no significant effect on CX_3CR1, IL-1β, and IL-4Rα (figure 7). These results show that aged microglia do not resolve inflammation compared to their adult counterparts.

3.8. TGFβ inhibition may cause prolonged inflammation

To understand the basal effect of TGFβ, adult mice were treated ICV with a TGFβ inhibitor following i.p. LPS injection. It is worth noting that this experiment is preliminary and another repeat is in preparation. Mice treated with both LPS and TGFβ inhibitor displayed social exploratory behavior at 50% of baseline 24 h after LPS ($p < 0.03$). This is significantly lower than all other experimental groups that established approximately 100% of baseline at the same time point (figure 8a). Analysis of brain slice mRNA showed a tendency increased IL-1β, IL-6, and TNFα expression ($p < 0.2$ for each) and a significant decrease in CX_3CR1 ($p < 0.03$) in LPS- and TGFβ inhibitor-treated mice (figure 8b). Microglia-specific mRNA analysis showed a tendency of increased CX_3CR1 ($p < 0.09$) and IL-1β ($p < 0.1$) (figure 8c). These preliminary results suggest that TGFβ may be important in hindering inflammatory cytokine expression that

correlates with sickness behavior.

4. Discussion

Microglia are the innate immune cell in the CNS and serve to respond to infection and other immune stimuli. Upon activation by immune stimuli, microglia release pro-inflammatory cytokines to regulate an adaptive sickness response. Regulation of microglia by anti-inflammatory cytokines may be necessary for the transition to a reparative M2 phenotype. Aged microglia, however, exhibit a more inflammatory phenotype characterized by exaggerated and prolonged expression of inflammatory markers and maladaptive sickness behavior. Primed microglia in the aged brain may explain the comorbidity of depression and cognitive problems with peripheral illnesses and injury in the elderly. Therefore, the regulation of aged microglia is of great interest.

The purpose of this study was to evaluate the extent to which several anti-inflammatory cytokines (IL-4, IL-10, and TGFβ) can regulate both adult and aged microglia. To determine these effects, a panel of pro- and anti-inflammatory markers was selected. This panel includes arginase, CX_3CR1, IL-1β, IL-4Rα, IL-6, iNOS, TNFα, SOCS1 and SOCS3.

This study began by determining the effects of IL-4, IL-10, or TGFβ treatment on activated BV2 or primary microglia. The results of these experiments indicate that IL-4 and TGFβ may be more potent anti-inflammatory mediators of microglia than IL-10. The discovery that microglia are less responsive to IL-10 than IL-4 and TGFβ was surprising considering the anti-inflammatory nature of IL-10 in the periphery. In addition, flow cytometry analysis showed that LPS-activated enriched microglia up-regulated IL-4Rα, but not IL-10R1, at 4 h and 24 h post LPS. These data may explain why LPS-activated microglia are less responsive to IL-10 than IL-4. Following these experiments, the study focused on IL-4 and TGFβ treatments in the context of aging.

In the aging experiments, adult and aged LPS-activated microglia were treated *ex vivo* with IL-4 or TGFβ. The results of IL-4 treatment on adult microglia show a similar anti-inflammatory phenotype as expressed in the IL-4 treated BV2 cultures. One notable difference is that IL-4 treatment had no significant effect on IL-1β mRNA expression in adult microglia. In addition, aged LPS-activated microglia showed a decreased capacity to resolve inflammation compared to adults. This unresolved inflammatory state was characterized by increased IL-1β expression, no reduction in iNOS expression, and an increase in arginase expression that was significantly less than the induction of arginase in adult LPS-activated microglia. Aged microglia were unable to properly regulate inflammation with IL-4 treatment compared to adults. To better explain this phenomenon, adult and aged LPS-activated microglia expression of IL-4Rα was analyzed using flow cytometry. These results showed that there was no induction of IL-4Rα 4 h or 24 h after LPS in aged microglia; in comparison, adult microglia up-regulated IL-4Rα expression both 4 h and 24 h after LPS. These data may explain the lack of inflammation resolution in aged microglia treated *ex vivo* with IL-4. Moreover, these findings support the notion that aged microglia are unresponsive to anti-inflammatory regulation.

In addition, to further investigate microglia regulation of inflammation in normal aging, adult and aged LPS-activated microglia were treated *ex vivo* with TGFβ. These results show a similar anti-inflammatory profile as expressed in the TGFβ treated BV2 and primary cultures. One exception is that *ex vivo* TGFβ treatment had no effect on CX_3CR1 expression and this was unexpected when considering previous studies that show induction of CX_3CR1 with TGFβ treatment (Wynne et al., 2010). It is noteworthy, however, that TGFβ inhibitors were unable to attenuate the induction of CX_3CR1; this may indicate that TGFβ alone is not responsible for these effects on CX_3CR1 expression. Furthermore, LPS-activated microglia from aged mice did

not respond to TGFβ treatment. These results show that activated aged microglia are do not respond to IL-4 and TGFβ in the same anti-inflammatory manner that adult microglia respond.

Additionally, to understand the basal effects of TGFβ, adult mice were injected i.p. with LPS then injected ICV with a TGFβ inhibitor. Social exploratory behavior analysis showed that at 24 h after LPS, the mice treated with LPS and TGFβ inhibitor still maintained social withdrawal behavior. This effect was significantly different from mice treated with LPS alone. As such, this signifies that TGFβ may be necessary in regulating other cytokines (IL-1β, IL-6, and TNFα) responsible for maintaining sickness behavior. Supporting this idea, LPS-activated microglia treated with TGFβ inhibitor had a tendency to increase expression of IL-1β; analysis of brain slice mRNA from this group showed a tendency for increased expression of IL-1β and IL-6. Furthermore, previous studies show increased TGFβ in the brains of adult but not aged mice 24 h after LPS (Wynne et al., 2010). Therefore, the results of this TGFβ inhibitor experiment may explain the lack of response in aged LPS-activated microglia to TGFβ regulation.

Figures

Figure 1

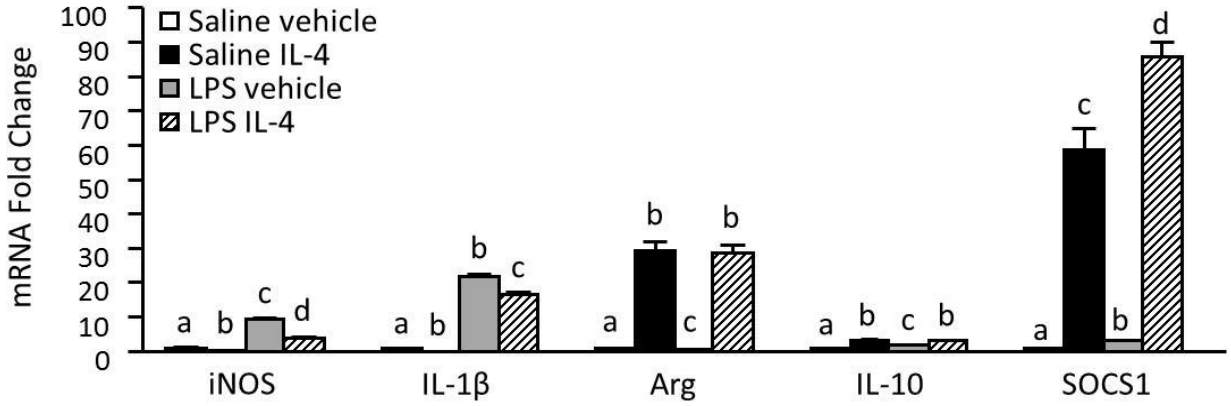

Figure 2a

BV2 mRNA Fold Change				
	Saline	Saline-TGFβ	LPS	LPS-TGFβ
CX3CR1	1.00 ± 0.04[a]	4.10 ± 0.04[b]	2.57 ± 0.18[c]	10.08 ± 0.64[d]
IL-4Rα	1.02 ± 0.09[a]	6.39 ± 1.56[b]	0.49 ± 0.22[c]	1.66 ± 0.60[d]
IL-1β	1.01 ± 0.06[a]	0.20 ± 0.01[b]	35.04 ± 4.08[c]	4.70 ± 0.72[d]
IL-6	1.00 ± 0.03[a]	0.86 ± 0.09[a]	77.40 ± 7.07[b]	13.99 ± 2.29[c]
TNFα	1.00 ± 0.01[a]	0.76 ± 0.06[a]	9.35 ± 0.53[b]	5.64 ± 0.71[c]
IL-10	1.00 ± 0.04[a]	0.21 ± 0.01[b]	1.41 ± 0.12[c]	0.57 ± 0.06[d]
SOCS3	1.02 ± 0.12[a]	1.38 ± 0.32[a]	6.01 ± 0.67[b]	5.12 ± 0.43[b]

Figure 2b

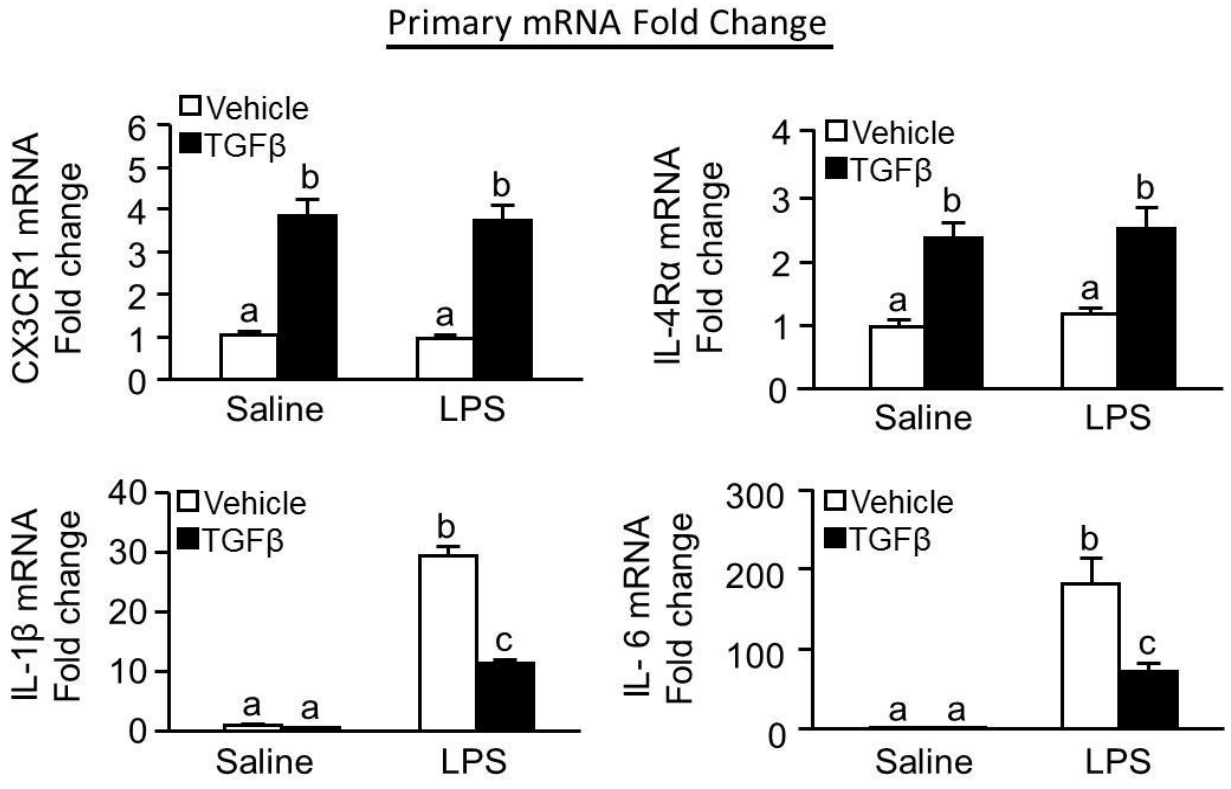

Figure 3

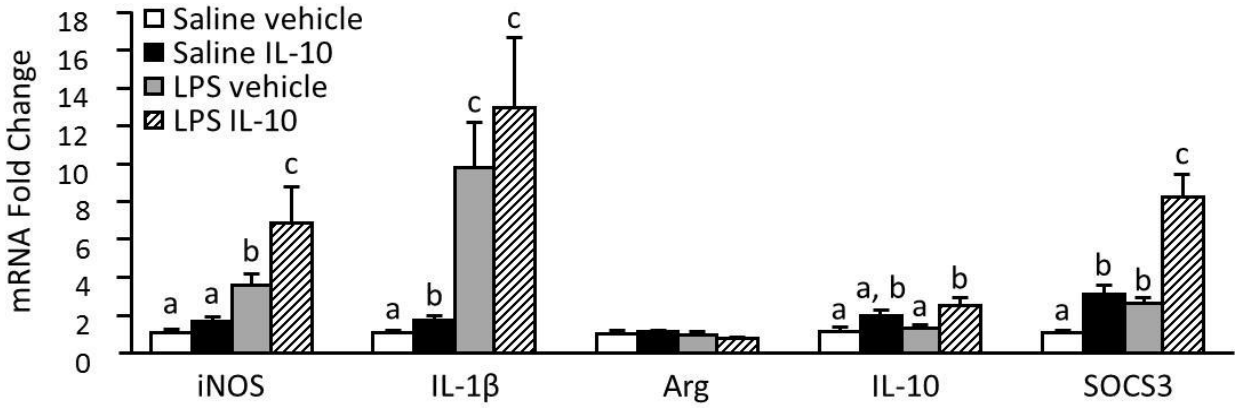
mRNA Fold Change
18
16
14
12
10
8
6
4
2
0
Saline vehicle
Saline IL-10
LPS vehicle
LPS IL-10
a
a
b
c
a
b
c
c
a
a, b
a
b
a
b
b
c
iNOS
IL-1β
Arg
IL-10
SOCS3

Figure 4

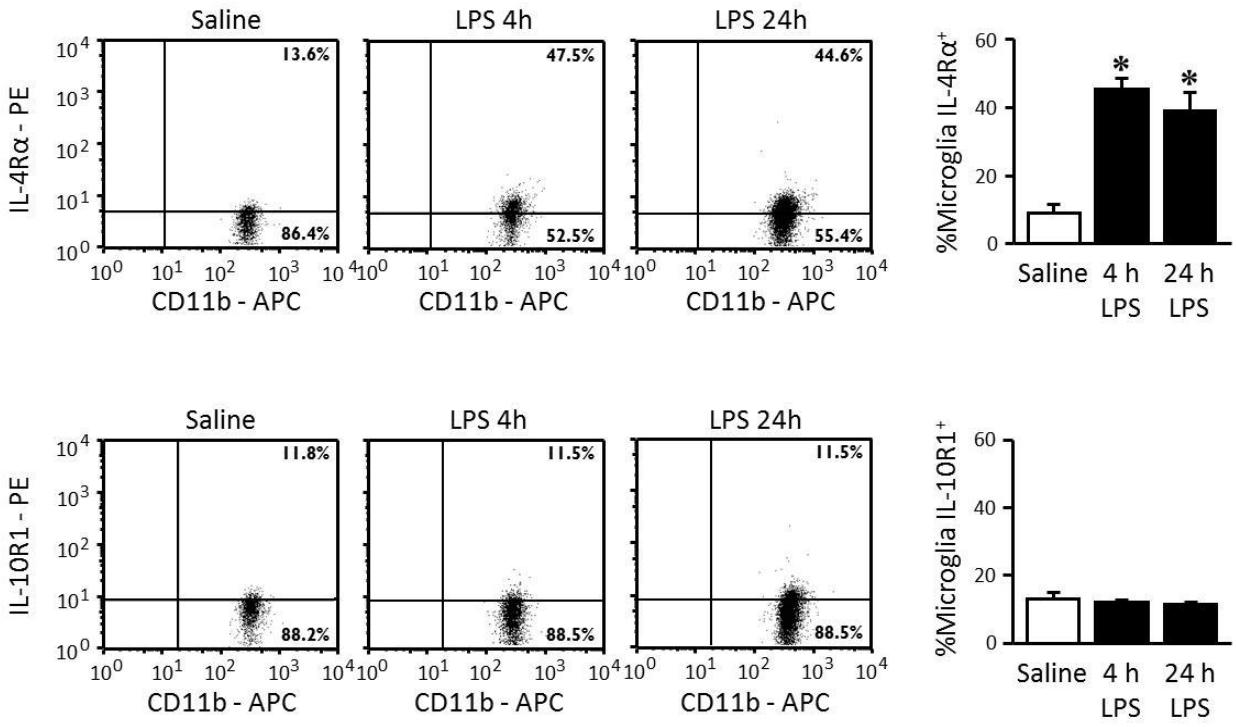
Saline
LPS 4h
LPS 24h
IL-4Rα - PE
13.6%
86.4%
47.5%
52.5%
44.6%
55.4%
CD11b - APC
%Microglia IL-4Rα+
Saline
4 h LPS
24 h LPS
IL-1OR1 - PE
11.8%
88.2%
11.5%
88.5%
11.5%
88.5%
%Microglia IL-1OR1+

Figure 5

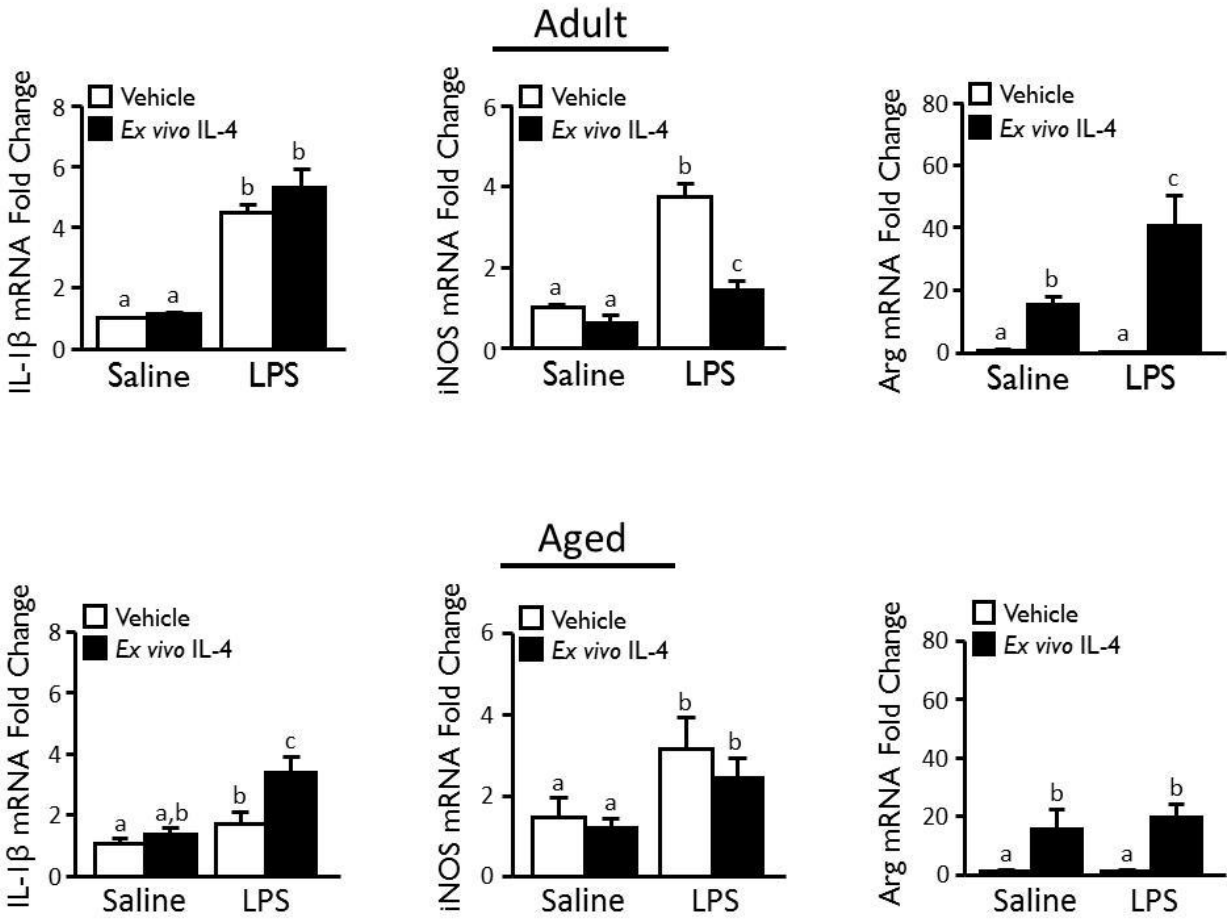
Adult
Aged
Vehicle
Ex vivo IL-4
IL-1β mRNA Fold Change
iNOS mRNA Fold Change
Arg mRNA Fold Change
Saline
LPS

Figure 6

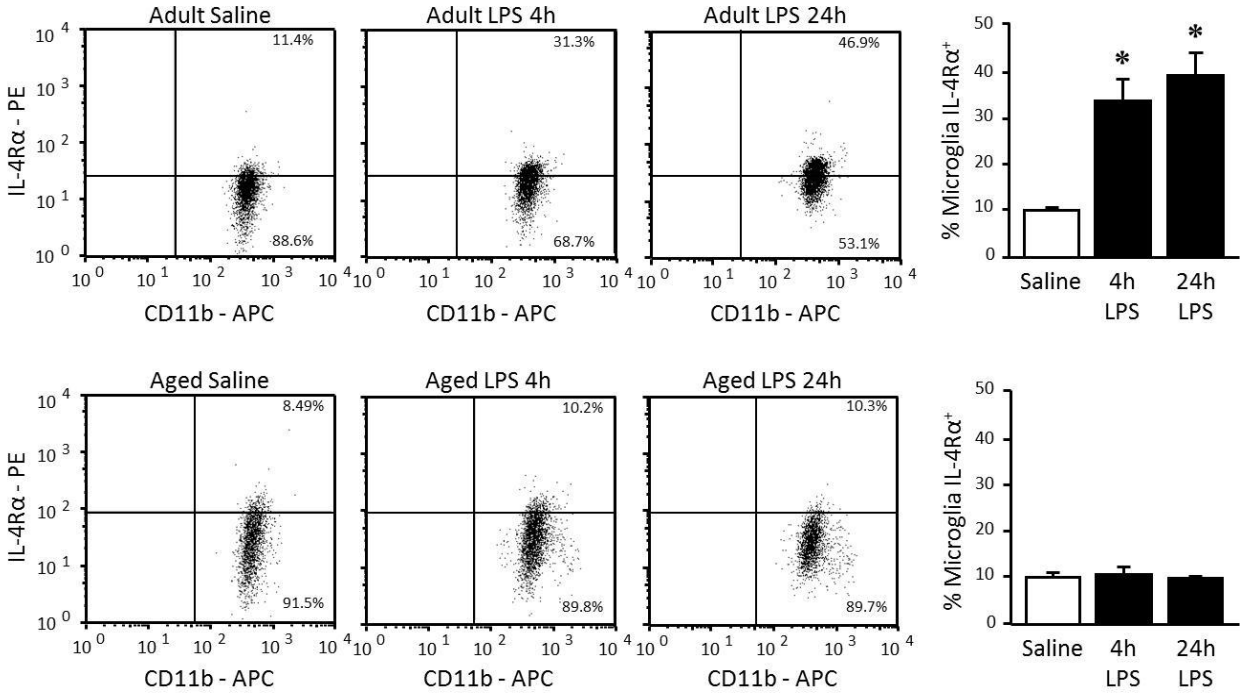
Adult Saline
Adult LPS 4h
Adult LPS 24h
11.4%
88.6%
31.3%
68.7%
46.9%
53.1%
IL-4Rα - PE
CD11b - APC
% Microglia IL-4Rα+
Saline
4h LPS
24h LPS
Aged Saline
Aged LPS 4h
Aged LPS 24h
8.49%
91.5%
10.2%
89.8%
10.3%
89.7%

Figure 7

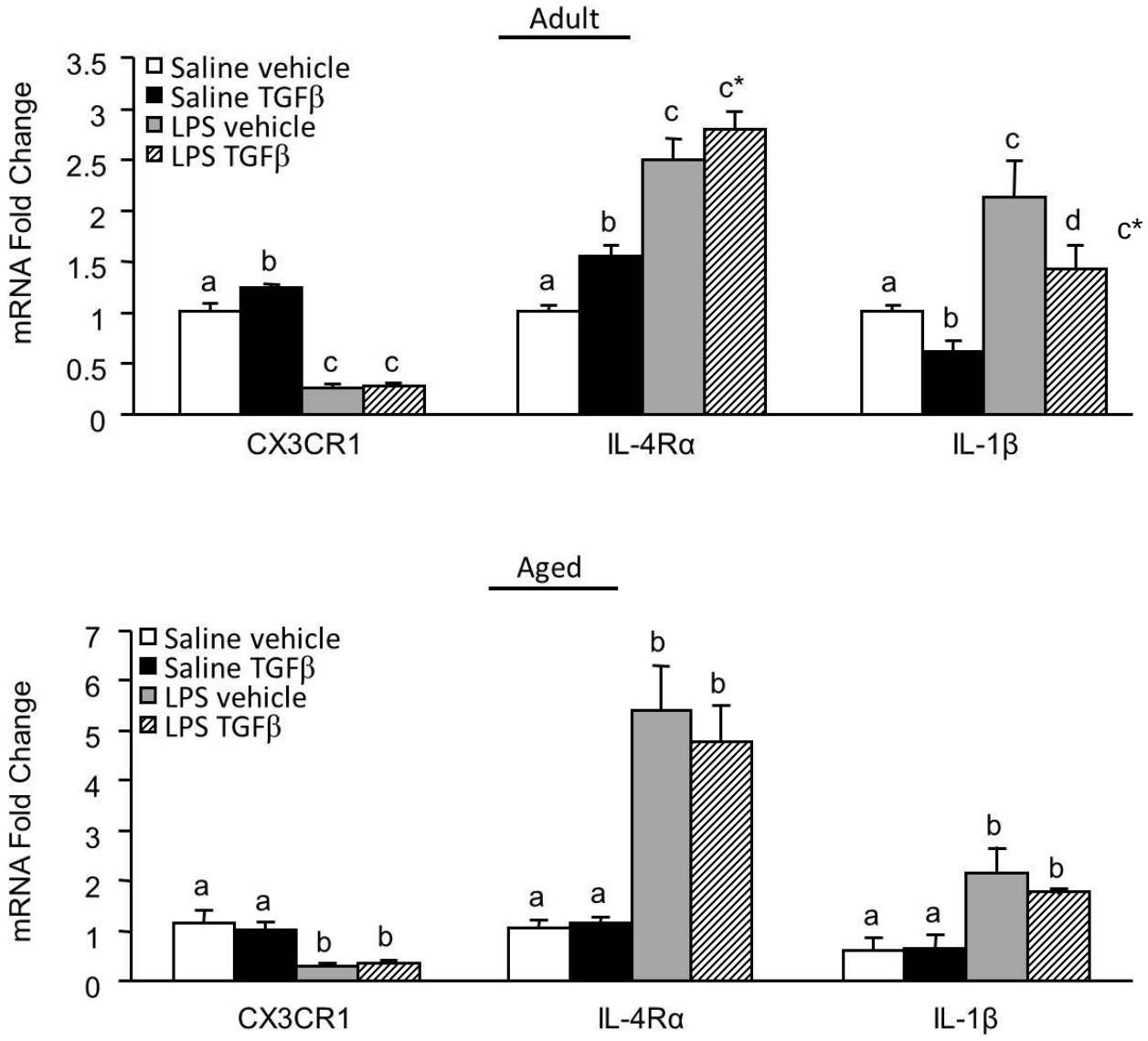
Adult
Saline vehicle
Saline TGFβ
LPS vehicle
LPS TGFβ
mRNA Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
c
a
b
c
c*
a
b
c
d
c*
CX3CR1
IL-4Rα
IL-1β
Aged
Saline vehicle
Saline TGFβ
LPS vehicle
LPS TGFβ
mRNA Fold Change
7
6
5
4
3
2
1
0
a
a
b
b
a
a
b
b
a
a
b
b
CX3CR1
IL-4Rα
IL-1β

Figure 8a

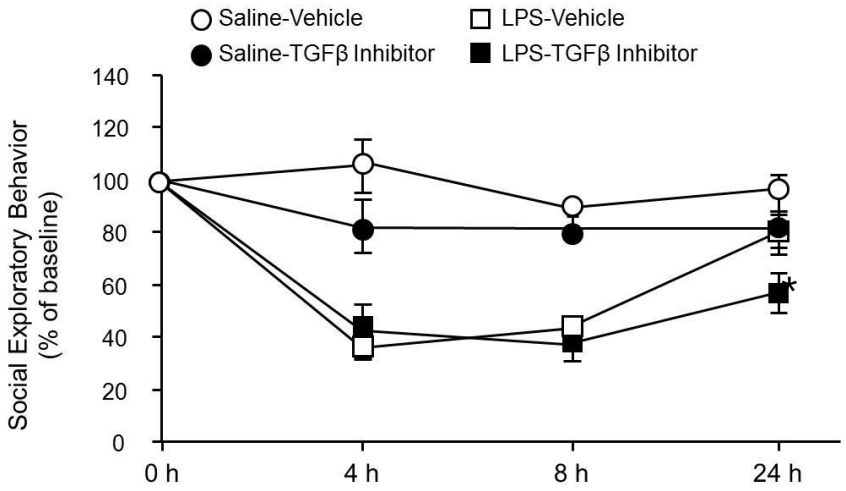

Figure 8b

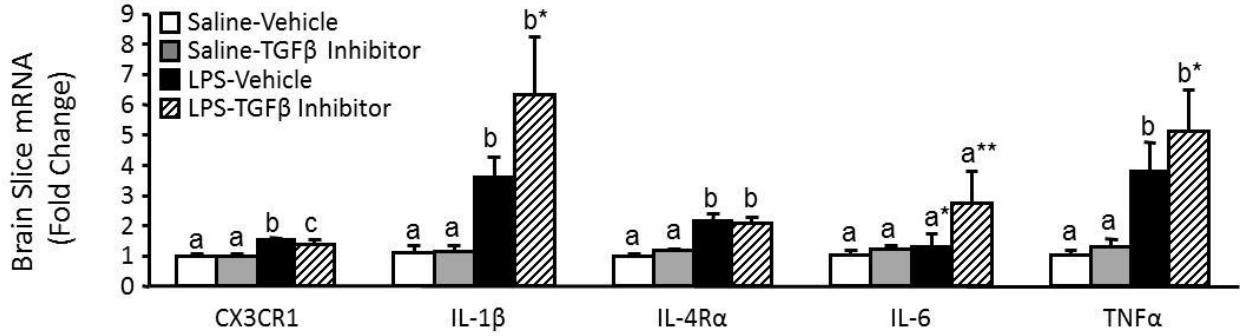

Figure 8c

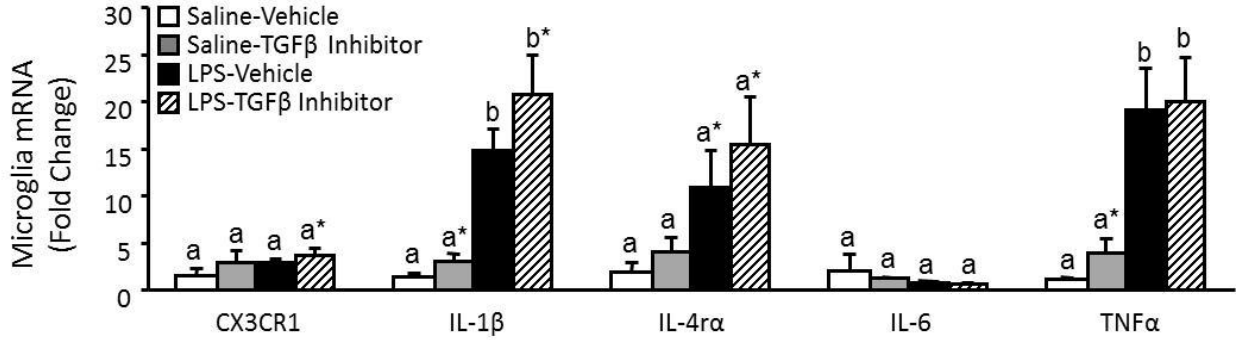

Figure Legends

Figure 1. IL-4 treatment down-regulated M1-related genes and up-regulated M2-related genes in BV2 microglia. BV2 cells were seeded 100,000 cells per well on a 24-well plate, pre-treated with saline or LPS (10 ng/ml) for 1h, and then treated with vehicle (0.1% BSA) or IL-4 (20 ng/mL) for 3 h. RNA was isolated by TriReagent separation, reverse transcribed to cDNA, and used for RT-PCR to determine the expression of M1-related (iNOS, IL-1β) and M2-related (arginase, IL-10,SOCS3) genes. Bars represent the mean ± SEM. Means with different letters (a, b, c, d) are significantly ($p \leq 0.05$) different from each other. n = 6

Figure 2. TGFβ decreases inflammatory cytokine expression in activated BV2 and primary microglia. BV2 microglia or primary microglia from neonatal mice were treated with saline or LPS (100 ng/ml for BV2 cells; 10 ng/ml for primaries). After 1 h, TGFβ (1 ng/ml) or vehicle (0.1% BSA) was added and microglia were incubated for an additional 3 h. RNA was collected and reverse transcribed to cDNA which was then analyzed using qPCR. Bars represent the mean ± SEM. Means with different letters (a, b, c, d) are significantly different ($p \leq 0.05$) from each other. n = 6

Figure 3. IL-10 failed to fully promote an M2 profile in activated BV2 microglia. BV2 cells were seeded 100,000 cells per well on a 24-well plate, pre-treated with saline or LPS (10 ng/ml) for 1h, and then treated with vehicle (0.1% BSA) or IL-10 (10 ng/mL) for 3 h. RNA was isolated by TriReagent separation, reverse transcribed to cDNA, and used for RT-PCR to determine the expression of M1-related (iNOS, IL-1β) and M2-related (arginase, IL-10, SOCS1) genes. Bars represent the mean ± SEM. Means with different letters (a, b, c, d) are significantly ($p \leq 0.05$) different from each other. n = 6

Figure 4. Peripheral LPS injection increased surface expression of IL-4Rα, but not IL-10R1, on adult microglia. Adult (3-6 mo) BALB/c mice received an intraperitoneal (i.p.) injection of saline or LPS (0.33 mg/kg) and euthanized either 4 h or 24 h later. Microglia were isolated by Percoll density gradient and stained for IL-4Rα, IL-10R1 CD45, and CD11b for analysis of protein expression by flow cytometry. Microglia were identified as CD11b+/CD45^{low}. Bars represent the mean ± SEM. Means with * are significantly ($p \leq 0.05$) different from saline control. n = 4

Figure 5. Ex vivo IL-4 treatment lowered inflammation in adult, but not aged, microglia. Adult (3-6 mo) and aged (18-22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and euthanized 4 h later. Microglia were isolated by Percoll density gradient, plated for 1 h, and treated with IL-4 (20 ng/mL). RNA was isolated 3 h later, reverse transcribed to cDNA, and used for RT-PCR to determine the expression of IL-1β, iNOS, and arginase. Bars represent the mean ± SEM. Means with different letters (a, b, c) are significantly ($p \leq 0.05$) different from saline. n = 9

Figure 6. Peripheral LPS injection increased surface expression of IL-4Rα on adult, but not aged, microglia. Adult (3-6 mo) and aged (18-22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and euthanized either 4 h or 24 h later. Microglia were isolated by Percoll density gradient and stained for IL-4Rα, CD45, and CD11b for analysis of protein expression by flow cytometry. Microglia were identified as CD11b+/CD45^{low}. Bars represent the mean ± SEM. Means with * are significantly ($p<0.05$) different from saline. n = 4

Figure 7. Resolution of inflammation is incomplete in aged microglia with TGFβ treatment. Adult (3 mo) and aged (20 mo) mice were injected i.p. with LPS (0.33 mg/kg). After 4 h,

microglia were isolated, plated and treated with TGFβ (1 ng/mL) or vehicle (0.1% BSA) for an additional 3 h. RNA was collected and reverse transcribed to cDNA which was then analyzed using qPCR. Bars represent the mean ± SEM. Means with different letters (a, b, c, d) are significantly different ($p \leq 0.05$) from each other. An asterisk (*) signifies a trend ($p \leq 0.10$). n = 5

Figure 8. TGFβ inhibition may cause prolonged inflammation. Adult BALB/c mice underwent surgery to install an injection cannula (bregma -1.2 mm laterally, -0.5 mm caudally). 1 week later, mice were injected i.p. with LPS (0.33 mg/kg) and after 30 mins ICV injected with vehicle (50/50 DMSO/saline) or the TGFβ inhibitor SB431542 (2.5 nmoles). Social exploratory behavior was pef formed at 0 h, 4 h, 8 h, and 24 h. After 24 h, microglia were isolated and a brain slice was collected. RNA was collected and reverse transcribed to cDNA which was then analyzed using qPCR. Bars represent the mean ± SEM. Means with different letters (a, b, c, d) are significantly different ($p \leq 0.05$) from each other. An asterisk (*) signifies $0.05 \leq p \leq 0.2$ 0.2. n = 3 to 8

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